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Dexamethasone counteracts the immunostimulatory effects of triiodothyronine (T3) on dendritic cells

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ABSTRACT

Glucocorticoids (GCs) are widely used as anti-inflammatory and immunosuppressive agents. Several studies have indicated the important role of dendritic cells (DCs), highly specialized antigen-presenting and immunomodulatory cells, in GC-mediated suppression of adaptive immune responses. Recently, we demonstrated that triiodothyronine (T3) has potent immunostimulatory effects on bone marrow-derived mouse DCs through a mechanism involving T3 binding to cytosolic thyroid hormone receptor (TR) β1, rapid and sustained Akt activation and IL-12 production. Here we explored the impact of GCs on T3-mediated DC maturation and function and the intracellular events underlying these effects. Dexamethasone (Dex), a synthetic GC, potently inhibited T3-induced stimulation of DCs by preventing the augmented expression of maturation markers and the enhanced IL-12 secretion through mechanisms involving the GC receptor. These effects were accompanied by increased IL-10 levels following exposure of T3-conditioned DCs to Dex. Accordingly, Dex inhibited the immunostimulatory capacity of T3-matured DCs on naive T-cell proliferation and IFN-γ production while increased IL-10 synthesis by allogeneic T cell cultures. A mechanistic analysis revealed the ability of Dex to dampen T3 responses through modulation of Akt phosphorylation and cytoplasmic-nuclear shuttling of nuclear factor-κB (NF-κB). In addition, Dex decreased TRβ1 expression in both immature and T3-maturated DCs through mechanisms involving the GC receptor. Thus GCs, which are increased during the resolution of inflammatory responses, counteract the immunostimulatory effects of T3 on DCs and their ability to polarize adaptive immune responses toward a T helper (Th)-1-type through mechanisms involving, at least in part, NF-κB- and TRβ1-dependent pathways. Our data provide an alternative mechanism for the anti-inflammatory effects of GCs with critical implications in immunopathology at the cross-roads of the immune-endocrine circuits.

1. Introduction

Glucocorticoids (GCs) are widely used as anti-inflammatory and immunosuppressive agents in many autoimmune and allergic diseases and in transplantation to prevent rejection. These steroid hormones are biological mediators naturally released during the course of inflammatory or stress responses [1]. Synthesis of GCs provokes a crucial negative feedback that limits the magnitude of immune responses, thus preventing potential damage to the host.

GCs affect growth, differentiation and function of many immune cell types, including T cells, macrophages, monocytes and dendritic cells (DCs). Most of their immunosuppressive effects are mediated through cytosolic ligand-inducible receptors [2]. Inactive GC receptors (GRs) are associated with heat shock proteins, which act as chaperones. Upon GC binding, this complex dissociates and the activated GR translocates to the nucleus where it binds to specific DNA motifs (GC-responsive elements) and to transcription factors such as activator protein 1 (AP1) and nuclear factor κB (NF-κB), thereby regulating the expression of a number of genes involved in the resolution of immune responses [3,4]. Through regulation of gene expression, GCs attenuate the production of pro-inflammatory mediators, including cytokines (interleukin 1 [IL-1], IL-2, tumor necrosis factor-α [TNF-α], interferon-γ

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[IFN-γ]), prostaglandins and nitric oxide. Moreover, GCs also inhibit expression of cell adhesion molecules involved in immune cell trafficking and may induce selective death of effector T cells [5–7].

The immunoregulatory activities of GCs have been primarily attributed to their ability to influence the T lymphocyte compartment [8]. However, it is now clear that they can also affect other cells within the immune system network. In this regard, several studies highlighted a main role for antigen-presenting cells (APCs) as mediators of GC-mediated immunosuppression. Among APCs, DCs are believed to be the most efficient cells capable of stimulating naive T lymphocytes and inducing antigen-specific immune responses [9]. Given the remarkable plasticity of these cells, manipulation of their function to favor the induction of DCs with immunogenic or tolerogenic properties could be exploited in order to stimulate or attenuate immune responses [10].

DCs can display two phenotypic stages, being either immature or mature which is eventually reflected by their different functional properties. DCs patrolling peripheral tissues which are in direct contact with the external environment are in an immature state (iDCs), scanning self and foreign antigens. In the absence of inflammatory stimuli, these cells exhibit high endocytic capacity and express low levels of major histocompatibility complex class II (MHC II) and co-stimulatory molecules. iDCs may be activated by inflammatory stimuli derived from tissue injury, necrotic cells or pathogens, leading to the generation of mature DCs (mDCs). During this process DCs up-regulate expression of the MHC II and co-stimulatory molecules, secrete a wide variety of pro-inflammatory cytokines, reduce the ability of taking up antigens, and augment their ability to stimulate T cells [11]. After *in vitro* or *in vivo* exposure to lipopolysaccharides (LPS) or other microbial products, DCs undergo activation and maturation through different signaling pathways including mitogen-activated protein kinase kinase 1 (MAPKK1)/extracellular signal-regulated kinase (ERK), which favors DC survival, and the Akt- and NF-κB pathways, which allow for DC maturation [12,13]. Signaling through NF-κB also determines the increased expression of MHC II and co-stimulatory molecules, release of pro-inflammatory cytokines and chemokines, and DC recruitment to secondary lymphoid organs. This coordinated process leads to sustained T-cell stimulatory capacity and IL-12 production, which results in the induction of protective T helper (Th)-1-type immunity [12].

GCs may influence DCs virtually at all levels of the differentiation or maturation process. While they stimulate antigen uptake, GCs can also suppress T cell activation through inhibition of MHC II, co-stimulatory molecules and cytokine expression. In addition, GCs not only suppress DC activity, they also endow DCs with tolerogenic properties through induction of IL-10 synthesis [14].

Thyroid hormones (THs) are essential for normal growth, development, differentiation and metabolism. The classic genomic actions of THs are mediated by nuclear TH receptors (TRs) that act as hormone-inducible transcription factors. TRs are encoded by two different gene loci, *TRA* and *TRB*. The *TRA* gene, located on chromosome 17, encodes one triiodothyronine (T3)-binding TRα1 and two splicing variants (TRα2 and TRα3). These TRα1 variants have no T3-binding activity. Truncated TRs, transcribed from an internal promoter, give rise to TRΔα1 and TRΔα2 that retain most of the T3-binding domain. Through alternative promoter usage, the *TRB* gene yields TRβ1, TRβ2, TRβ3 and the truncated variant TRΔβ3 which is unable to bind the thyroid hormone. The $TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$ and $TR\beta_3$ isoforms are widely expressed, whereas $TR\beta_2$ is predominantly restricted to the hypothalamus-pituitary axis. However, the notion of classical or genomic mechanisms as unique actions mediated by TRs has been challenged in the past decade by descriptions of TH actions that involve extranuclear (nongenomic) effects in a variety of cell types. These TH-dependent pathways are associated with extranuclear TRs localized within the cytoplasm and the plasma membrane and to TH-dependent effects mediated by the cell surface αβγ3 integrin. Several cytoplasmic T3 actions mediated by TRs are linked to activation of the phosphatidyl inositol 3-kinase (PI3K) pathway in alveolar cells and human fibroblasts. Moreover, activation of Akt, a critical component of cell growth and survival, has been detected in islet β cells upon engagement of $TR\beta_1$ and activation of PI3K-p85 [15].

A characteristic of T3 action is the multihormonal interaction in the final expression of a specific metabolic effect. Several reports indicated that T3 increases rat growth hormone synthesis in rat pituitary cell lines and that GCs synergistically stimulate T3 action at this level [16]. Diverse reports have also demonstrated that GCs increase the mRNAs of T3-regulated hepatic genes [17–19]. Dexamethasone (Dex), a synthetic GC, has been reported to potentiate T3-induced metamorphosis and increase TRs in Xenopus tadpole tails [20]. In this regard, we have demonstrated that Dex enhances T3-dependent actions in the liver through an increase of $TR\beta_1$ expression [21].

Recently, we provided the first evidence of the expression of TRs in both immature and LPS-matured bone marrow-derived mouse DCs, showing higher expression of TRβ1 than TRα1. Strikingly, the expression of cytoplasmic TRβ1 was markedly increased compared with that of nuclear TRβ1. Furthermore, we found that physiological levels of T3 stimulated the expression of DC maturation markers (MHC II, CD80, CD86 and CD40), markedly increased the secretion of IL-12, and stimulated the ability of DCs to induce naïve T cell proliferation and IFN-γ production in allogeneic T cell cultures [22]. Analysis of the mechanisms involved in these effects revealed the ability of T3 to increase Akt phosphorylation independently of PI3K activation, which was essential for supporting T3-induced DC maturation and IL-12 production. This effect was dependent on intact TRβ1 signaling as small interfering RNA-mediated silencing of TRβ1 expression prevented T3-induced DC maturation and IL-12 secretion as well as Akt activation. In turn, T3 up-regulated TRβ1 expression through mechanisms involving NF-κB signaling facilitating a positive regulatory loop to control hormone-dependent TRβ1 signaling [23].

In the present study we aim to examine the interplay between THs and GCs within the DC compartment and to analyze the molecular mechanisms underlying these biological effects.

2. Experimental

2.1. Mice

Female C57BL/6 (B6; H-2b) mice were obtained from Ezeiza Atomic Center (Buenos Aires, Argentina). Mice were maintained under specific pathogen-free conditions and used at 6–10 week-old. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH and the local institutional animal care committee.

2.2. DC preparation and culture

DCs were obtained as described by Inaba et al. [24]. Briefly, bone marrow progenitors were collected from the femurs of C57BL/6 mice, cultured in RPMI 1640 10% fetal calf serum (FCS) depleted of THs by treatment with resin AG-1-X8 (Bio-Rad), in the presence of GM-CSF from supernatant of J558 cell line and fed every 2 days. At day 8 of cell culture, >85% of the harvested cells expressed MHC II, CD40, CD80 and CD11c, but not Gr-1. iDCs were cultured with T3 (5 nM, DC_{T3}), lipopolysaccharides (LPS; 100 ng/mL; *Escherichia coli* strain 0111:B4; Sigma; DC_{LPS}), Dex (10 nM, DC_{Dex}) and RU486 (10^{-8} to 10^{-6} M, DC_{RU486}) for different time periods. Parallel cultures were maintained without stimuli and used as controls (DC).

T3 (3,3′,5-triiodo-L-thyronine), Dex and RU486 were purchased from Sigma Chemical Co. (USA) and prepared according to the manufacturer's recommended protocol.

2.3. Flow cytometric analysis of DC phenotype

DCs were washed twice with PBS supplemented with 2% (vol/vol) FCS and resuspended in 10% (vol/vol) FCS in PBS. Cells were then incubated with the following fluorochrome-conjugated monoclonal antibodies (mAbs) for 30 min at 4 °C: fluorescein isothiocyanate (FITC)-anti-CD11c, phycoerythrin (PE)-anti-IA/IE (MHC II), PE-anti-CD40, PE-anti-CD80, and PE-anti-CD86 (all from BD PharMingen, San Diego, California, USA). Cells were then processed and analyzed in an FACS canto II flow cytometer (BD Biosciences PharMingen, NJ, USA) using FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Cytokine determination

Intracellular cytokine detection was assessed by flow cytometry as described [25] using PE-conjugated anti-IL-12 mAb (BD Biosciences PharMingen, New Jersey, USA). Briefly, DCs incubated with T3 or LPS in presence of Dex, were exposed to brefeldin A (10 μg/ml; Sigma) for the last 4 h of cell culture. Cells were then fixed with 1% (vol/vol) paraformaldehyde, treated with FACS permeabilizing solution and stained with an optimal concentration of anti-cytokine mAb or an appropriate isotype control (all from BD Biosciences PharMingen). Cells (at least 10,000 viable cells) were then analyzed in FACS canto II flow cytometer (BD Biosciences PharMingen, New Jersey, USA) using Flowjo software (Tree Star, Ashland, OR).

IL-12p70, IL-10 and IFN-γ detection was performed in cell culture supernatants using standard capture enzyme-linked immunosorbent assays (ELISA) (BD Biosciences PharMingen, New Jersey, USA). Coating Abs included a rat anti-mouse IL-12p70 mAb (clone C15.6), rat anti-mouse IL-10 mAb (clone JES5–2A5) and rat anti-mouse IFN-γ mAb (clone R4–6A2). Detection Abs included biotinylated rat anti-mouse IL-12p70 mAb (clone C17.8), biotinylated rat anti-mouse IL-10 mAb (clone SXC-1) and biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2). Streptavidin-horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine were used as enzyme and substrate, respectively.

2.5. Allogeneic T cell cultures

Allogeneic T cell cultures were performed to assess the ability of DCs to stimulate allogeneic splenocytes *in vitro* as described [22]. Briefly, allogeneic splenocytes (1×10^5 cells/well, responder cells) were incubated for 3 days with irradiated DCs (30 Gy, stimulator cells) at a ratio of 1:15 (DC/splenocytes) in 96-well round-bottom plates. On day 2, 0.5 μCi (0.0185 MBq)/well of [3H]-thymidine (Amersham Life Sciences, Buckinghamshire, UK) was incorporated into each well for 18 h. Proliferation was determined as counts per minute of triplicate determinations.

2.6. Apoptosis assay

DC apoptosis was analyzed by flow cytometry following double labeling of cells with FITC-conjugated annexin V Ab and 7-amino-actinomycin D (7-AAD) as described [26].

2.7. Preparation of total, nuclear and cytoplasmic extracts

To obtain DC total cell lysates, 5×10^6 cells were resuspended in 100 μl of RIPA buffer and protease inhibitors, and incubated on ice for 30 min, followed by removal of DNA and cell debris by centrifugation at 10,000*g* for 20 min at 4 °C. Nuclear and cytoplasmic DC extracts were obtained by subcellular fractionation as described previously [27]. Briefly, 6×10^6 cells were resuspended in 200 μl of buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) Then, 25 μl of 10% (vol/vol) Nonidet P-40 were added and the solution was incubated on ice for 15 min. The supernatant containing cytoplasm was collected by centrifugation. The nuclear pellet was resuspended in 50 μl of ice-cold buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and the tube was vigorously rocked at 4 °C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at 4 °C, and the supernatant was collected. Total protein concentration was measured by the Bradford technique [28]. The reactions which involved Phospho-Akt immunodetection were stopped at the indicated times by adding cold saline solution and samples were centrifuged. Cell pellets were resuspended in loading buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 5% 2-ME) and boiled at 96 °C for 5 min. Aliquots were stored at −80 °C until use for Western blotting.

2.8. Western blotting

Antibodies directed to $TR\beta_1$ (sc-738 mouse monoclonal), NF-κB-p65 (sc-8008 mouse monoclonal), Actin (sc-8432 mouse monoclonal) and HRP-conjugated anti-mouse or anti-rabbit IgG were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, California, USA). Abs directed to Phospho-Akt (5473, rabbit monoclonal) and IκB-ε (611408, mouse monoclonal) was purchased from Cell Signaling Technology, Inc (Massachusetts, USA) and BD Biosciences (PharMingen, New Jersey, USA), respectively.

DC extracts were separated by SDS–PAGE, transferred to nitrocellulose membranes (Biorad Laboratories, Hercules, California, USA), and then blocked with 5% bovine albumin in PBS containing 0.05% Tween 20. Membranes were then blotted with Abs against $TR\beta_1$, NF-κB-p65, Actin, Phospho-Akt (1:1000 dilution) and IκB-ε (1:500 dilution) followed by HRP-conjugated anti-rabbit or anti-mouse IgG. Specific bands were developed by ECL (Amersham Life Sciences, Buckinghamshire, UK). Control of protein loading was revealed by actin immunodetection in the same blots.

2.9. Statistical analysis

Analysis of intergroup differences was conducted by one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test. *P* values less than 0.05 were considered statistically significant. All experiments were performed at least in triplicate.

3. Results

3.1. Dex prevents T3-induced DC maturation

To analyze the interplay between T3 and GCs in the control of immune cell functions we studied the effects of Dex on T3-induced DC maturation and function. For this, we first cultured iDCs with T3 (DC_{T3}) or LPS (positive control, DC_{LPS}) in the absence or the presence of Dex (DC_{Dex}) and evaluated the cell surface phenotype (MHC II, CD40, CD80 and CD86). As shown in Fig. 1, treatment of DCs with Dex alone (DC_{Dex}) did not alter the levels of CD40 and MHC II expression, yet it significantly inhibited expression of co-stimulatory molecules including CD80 (B7.1) and CD86 (B7.2). As expected, T3 and LPS both induced DC maturation, as demonstrated by the increased levels of MHC II, CD40, CD80 and CD86 on the

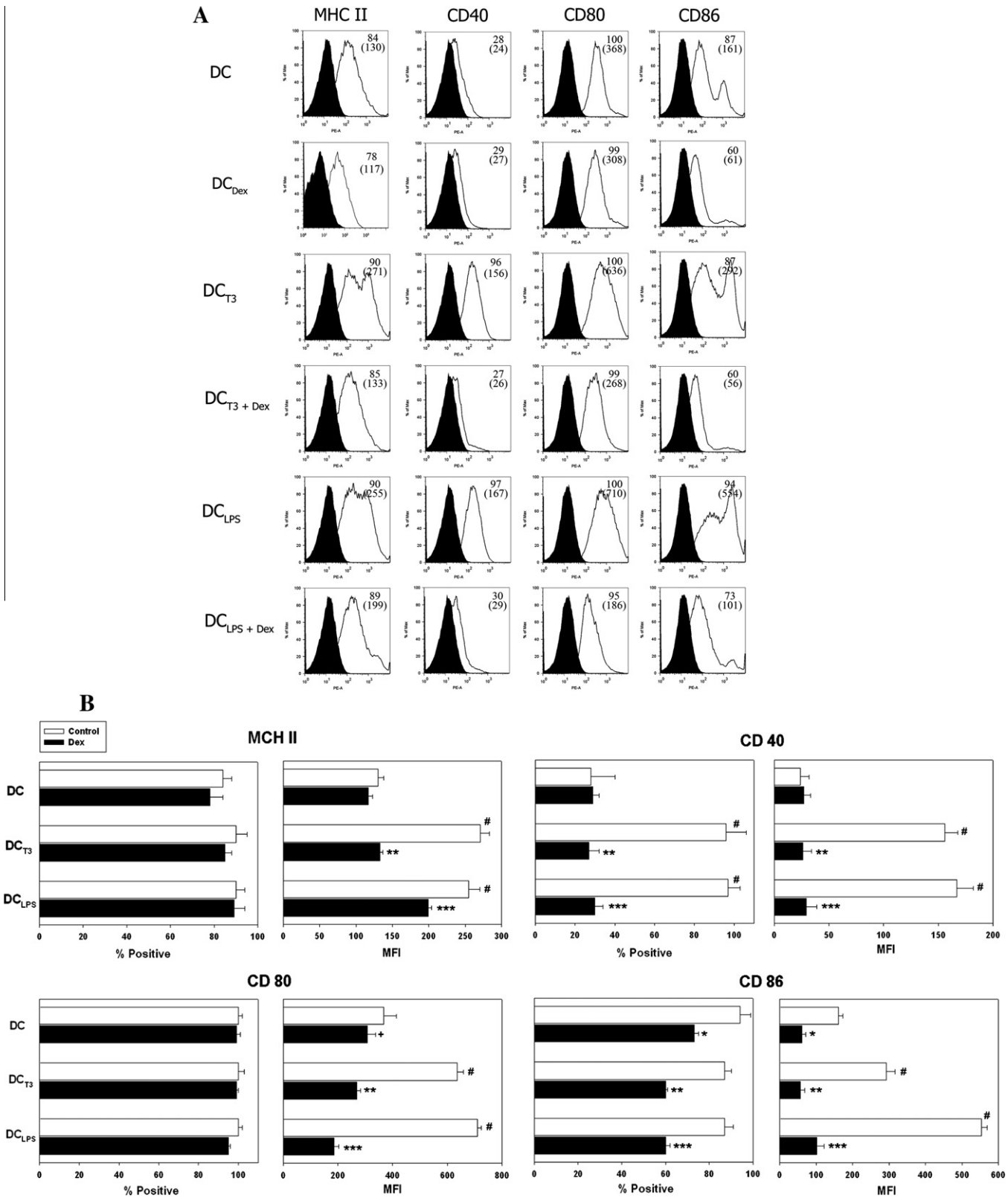

Fig. 1. Effect of Dex on cell surface phenotypic markers of T3-matured DCs. Bone marrow-derived iDCs differentiated with GM-CSF for 8 days (DC) were subjected to maturation with T3 (5 nM, DC_{T3}) or LPS (100 ng/ml, positive control, DC_{LPS}) in the absence or the presence of Dex (10 nM, DC_{Dex}) for 18 h. Cell surface phenotype was analyzed by flow cytometry of DC using PE-conjugated anti-MHC II (IA/IE), anti-CD40, anti-CD80 and anti-CD86 mAbs. (**A**) Representative histograms of three independent experiments are gated on $CD11c^+$ cells. Upper values represent percentages of positive cells, whereas lower values represent mean fluorescence intensity (MFI). Black histograms, nonspecific binding determined with isotype-matched control antibodies; white histograms, phenotypic markers. (**B**) Results are expressed as the percentage of positive cells (left panel) and MFI (right panel) within the $CD11c^+$ population. Data are expressed as mean ± SD and are from a representative experiment from a total of three with similar results, $^{\#}p < 0.001$, $^{*}p < 0.001$, $^{+}p < 0.05$ vs DC Control, $^{**}p < 0.001$ vs DC_{T3} Control; $^{***}p < 0.001$ vs DC_{LPS} Control.

surface of bone marrow-derived DCs. Addition of Dex and T3 to DC cultures resulted in downregulation of MHC II and co-stimulatory molecules. As previously reported, LPS-matured DCs showed decreased expression of MHC II, CD40, CD80 and CD86 when exposed to Dex [29]. These results indicate an inhibitory role of GCs in T3-induced DC maturation.

3.2. Dex controls the balance of pro- and anti-inflammatory cytokines in T3-matured DC

To investigate whether the inhibition in the maturation phenotype induced by Dex in T3-matured DCs was associated with a modified cytokine profile, we examined the ability of Dex to modulate cytokine production induced by T3. As expected, DCs exposed to T3 and LPS showed a significant increase in the frequency of IL-12-producing CD11c$^+$ DCs (Fig. 2A and B). Exposure to maturing DCs to Dex significantly suppressed their ability to produce IL-12 in response to T3 or LPS. To determine whether this effect was mediated by the steroid hormone receptor GR, T3 or LPS-matured DCs were pretreated with the GR antagonist RU486. As shown in Fig. 2A and B, Dex-inhibition of pro-inflammatory cytokine production induced by T3 or LPS was mediated through GR as RU486 hindered Dex-suppression of IL-12 production induced by either T3 or LPS. Consistently, Dex treatment induced a significant decrease in IL-12 secretion in DC cultures exposed to both T3 and LPS (Fig. 2C). In contrast, the anti-inflammatory cytokine IL-10 was higher in T3- and LPS- treated DCs incubated with Dex when compared to control DCs or DCs matured with T3 or LPS in the absence of Dex (Fig. 2D).

3.3. Dex abrogates the T-cell allostimulatory capacity of T3-matured DCs

Given the inhibitory effects of Dex on T3- or LPS-matured DCs, we assessed their allostimulatory capacity. As expected, proliferation of BALB/c (H-2d) splenocytes was strongly enhanced in response to co-culture with irradiated T3- and LPS-conditioned DCs (C57BL/6) (Fig. 3). Of note, DC treatment with Dex alone did not alter splenocyte proliferation when compared with control DCs. In contrast, the presence of Dex eliminated the ability of T3- or LPS-matured DCs to induce splenocyte proliferation.

As GCs have been reported to induce DC apoptosis under certain conditions [30] we examined whether the recorded effect was the result of Dex-induced DC death. For this purpose, we stained Dex/

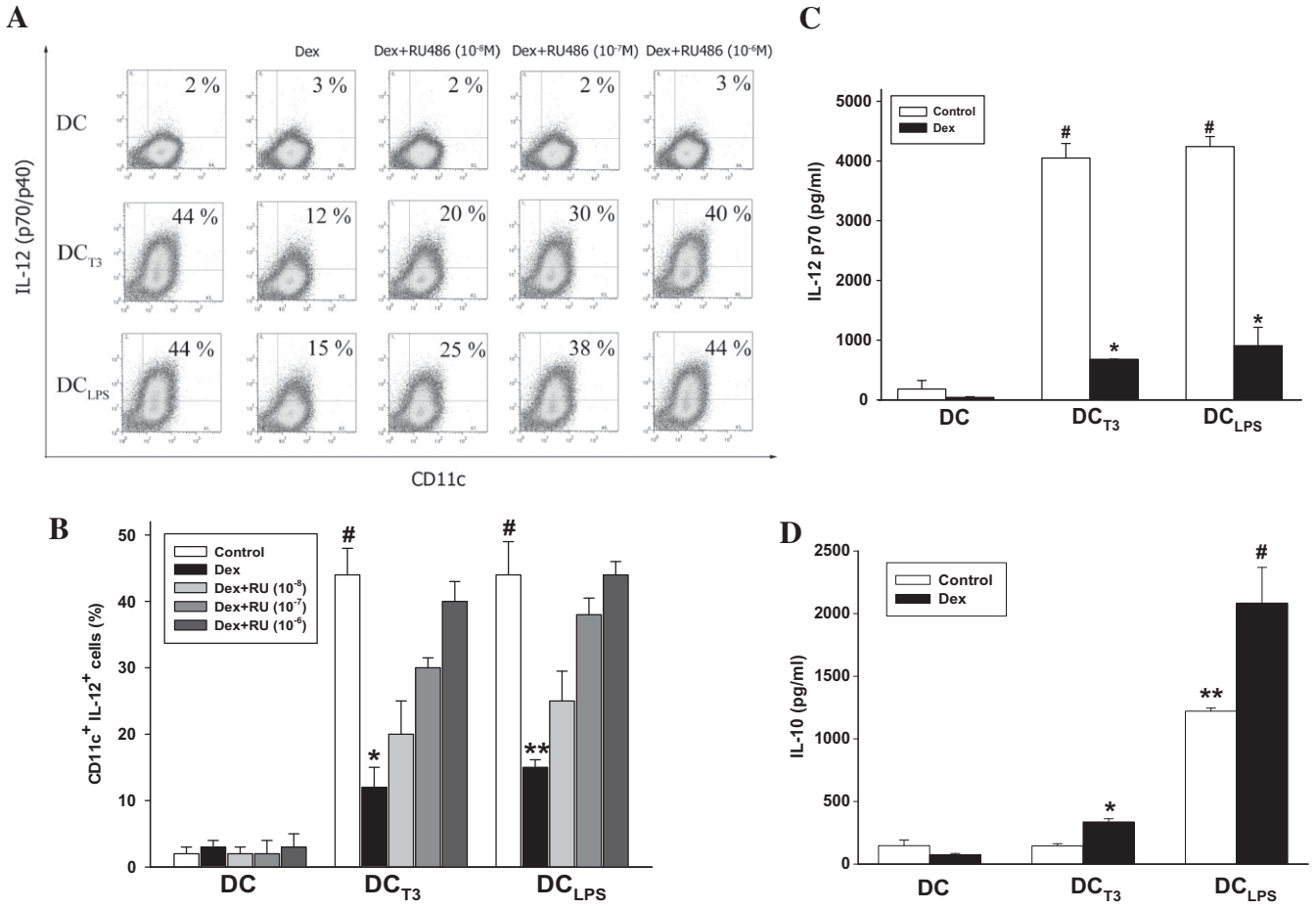

Fig. 2. Effect of Dex in the modulation of IL-12/IL-10 cytokine balance in T3-matured DCs. Bone marrow-derived iDCs differentiated with GM-CSF for 8 days (DC) were subjected to maturation with T3 (5 nM, DC_{T3}) or LPS (positive control, 100 ng/ml, DC_{LPS}) in the absence or the presence of Dex (10 nM, DC_{Dex}) or Dex + RU486 (steroid hormone antagonist, 10^{-8} to 10^{-6} M) for 18 h. (**A** and **B**) For intracytoplasmic cytokine staining, cells were incubated with brefeldin A for 4 h, stained for CD11c, fixed, permeabilized, and then stained with anti-IL-12p70 mAb as described in Section 2. The frequency of CD11c$^+$ IL-12$^+$ cells was determined by flow cytometry. (**A**) Representative intracytoplasmic IL-12 flow cytometric analysis. (**B**) Values are given as the percentage of total CD11c$^+$ IL-12-producing cells. Data are expressed as mean ± SD and are from a representative experiment from a total of three with similar results, $^{\#}p < 0.001$ vs DC Control, $^{*}p < 0.001$ vs DC_{T3} Control, $^{**}p < 0.001$ vs DC_{LPS} Control. (**C**) IL-12p70 production was determined in culture supernatants by ELISA. Data are expressed as mean ± SD (pg/ml) and are from a representative experiment from a total of three with similar results, $^{\#}p < 0.001$ vs DC Control; $^{*}p < 0.001$ vs DC_{T3} Control, $^{**}p < 0.001$ vs DC_{LPS} Control. (**D**) IL-10 production was determined in culture supernatants by ELISA. Data are expressed as mean ± SD (pg/ml) and are from a representative experiment from a total of three with similar results, $^{**}p < 0.01$ vs DC Control; $^{*}p < 0.01$ vs DC_{T3} Control, $^{\#}p < 0.01$ vs DC_{LPS} Control.

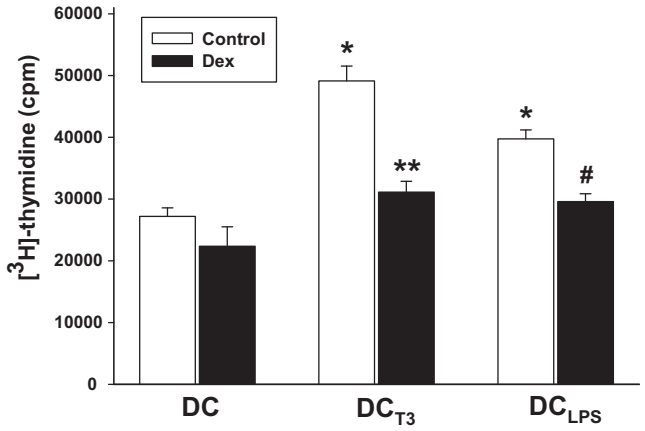

Fig. 3. Impact of Dex on the allostimulatory capacity of T3-matured DCs: effects on splenocyte proliferation. Bone marrow-derived iDCs were stimulated with T3 (5 nM, DC_{T3}) or LPS (positive control, 100 ng/ml, DC_{LPS}) in the absence or the presence of Dex (10 nM). After 18 h, DCs were extensively washed, irradiated, and cultured with allogeneic splenocytes for 3 days at 1:15 stimulator/responder (DC/splenocyte) ratio. Proliferation of allogeneic splenocytes was quantified by [^{3}H-thymidine] uptake. Data are expressed as mean ± SD (cpm) and are from a representative experiment from a total of three experiments with similar results, $^{*}p < 0.001$ vs DC Control; $^{**}p < 0.001$ vs DC_{T3} Control, $^{\#}p < 0.01$ vs DC_{LPS} Control.

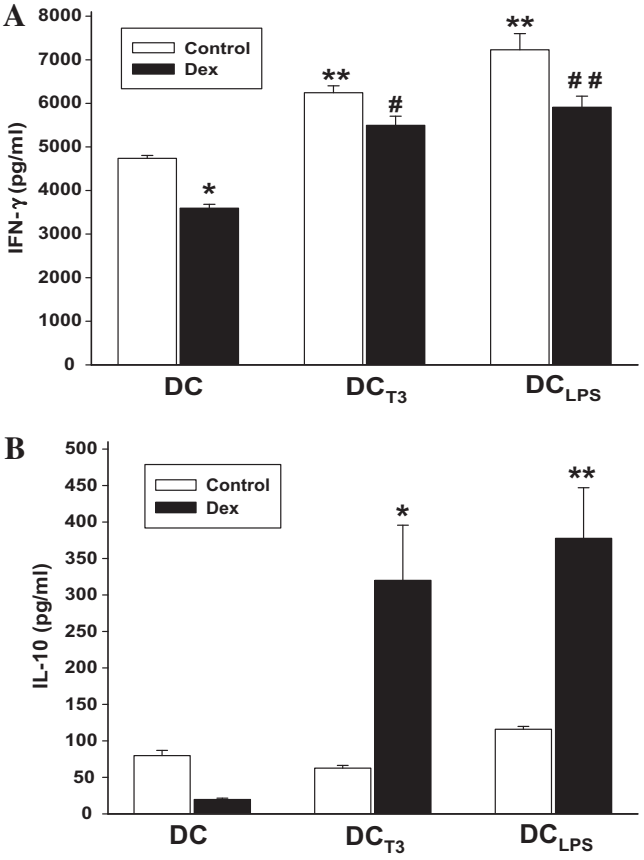

Fig. 4. Influence of Dex on the allostimulatory capacity of T3-conditioned DCs: effects on splenocyte-derived cytokine secretion. Bone marrow-derived iDCs were stimulated with T3 (5 nM, DC_{T3}) or LPS (positive control, 100 ng/ml, DC_{LPS}) in the absence or the presence of Dex (10 nM). After 18 h, DC were extensively washed, irradiated, and cultured with allogeneic splenocytes for 3 days at 1:15 stimulator/responder (DC/splenocyte) ratio. IFN-γ (**A**) and IL-10 (**B**) production were measured in culture supernatants by ELISA. (**A**) Results are expressed as mean ± SD (pg/ml) from a representative experiment from a total of three with similar results, $^{*}p < 0.01$; $^{**}p < 0.01$ vs DC Control; $^{\#}p < 0.05$ vs DC_{T3} Control; $^{\#\#}p < 0.01$ vs DC_{LPS} Control. (**B**) Results are expressed as mean ± SD (pg/ml) from a representative experiment from a total of three with similar results, $^{*}p < 0.001$ vs DC_{T3} Control; $^{**}p < 0.001$ vs DC_{LPS} Control.

T3-matured DCs with anti-annexin-V Ab and 7-AAD to detect early apoptotic cells. Remarkably, Dex did not increase the percentage of apoptotic cells over the 18 h-treatment period examined (data not shown), suggesting that inhibition of splenocyte proliferation induced by Dex/T3-treated DCs was not due to increased apoptosis or DC death.

In turn, reduction of the allostimulatory capacity of T3- and LPS-matured DCs induced by Dex was also evidenced by the lower production of the effector cytokine IFN-γ in culture supernatants of splenocytes co-cultured with Dex and T3- or LPS-conditioned DCs (Fig. 4A). In agreement, secretion of IL-10 was substantially increased on T cells co-cultured with allogeneic T3- or LPS-conditioned DCs in the presence of Dex (Fig. 4B). These data indicate that Dex counteracts the immunostimulatory capacity of DCs matured in the presence of T3.

3.4. Dex prevents T3-induced Akt activation

As T3 induces Akt phosphorylation which in turn favors maturation and T-cell stimulatory function of DCs [23] and these effects are diminished in the presence of Dex, we then investigated the effects of Dex on T3-induced Akt activation. Phosphorylation of Akt was examined in total cellular extracts from DCs exposed at various time points (5, 15, 30, 60 min and 18 h) to T3 stimulation in the presence or the absence of Dex. As expected, a significant increase in Ser 473 phosphorylation of Akt was detected as early as 5 min following exposure of DCs to T3 with a peak at 15 min of incubation. The increase in Akt phosphorylation persisted even at 18 h following addition of the thyroid hormone (Fig. 5A and B). The presence of Dex not only delayed the kinetics of T3-induced Akt phosphorylation, as the peak was detected at 30 min of incubation (Fig. 5A and B), but it also induced less phosphorylation at all time points analyzed when compared with T3 alone. Interestingly, Akt activation in Dex/T3-stimulated DCs reached levels comparable to control DCs after 18 h of cell culture.

3.5. Dex inhibits $TR\beta_1$ expression on DC through a GR-dependent mechanism

Given the ability of GCs to modulate TH actions through TR modulation [21], the positive effects of T3 on $TR\beta_1$ expression and the pivotal role of $TR\beta_1$ on the stimulatory effects of T3 and Akt phosphorylation [23], we evaluated the effects of Dex on $TR\beta_1$ expression in T3-matured DCs. Dex treatment of both iDCs (DC) and DCs matured with LPS (DC_{LPS}) led to a significant decrease in the expression of $TR\beta_1$ (Fig. 6A and B). Furthermore, Dex-induced inhibition of $TR\beta_1$ expression was abolished by RU486, suggesting involvement of GR leveling this regulatory effect. Likewise, Dex prevented T3-induced up-regulation of $TR\beta_1$ through a mechanism involving the GR.

3.6. Dex attenuates T3-induced cytoplasmic-nuclear shuttling of the NF-κB transcription factor

Since T3 induces cytoplasmic-nuclear shuttling of the NF-κB transcription factor to modulate DC functionality [22] and the NF-κB pathway is directly involved in $TR\beta_1$ expression [23], we next evaluated the effects of Dex on cytoplasmic and nuclear levels of NF-κB-p65 and IκB-ε in T3-matured DCs. In agreement with our previous report, T3 increased the presence of NF-κB-p65 in the nucleus and significantly reduced cytoplasmic NF-κB-p65 as well as total IκB-ε levels (Fig. 7A and B). Addition of Dex abolished these

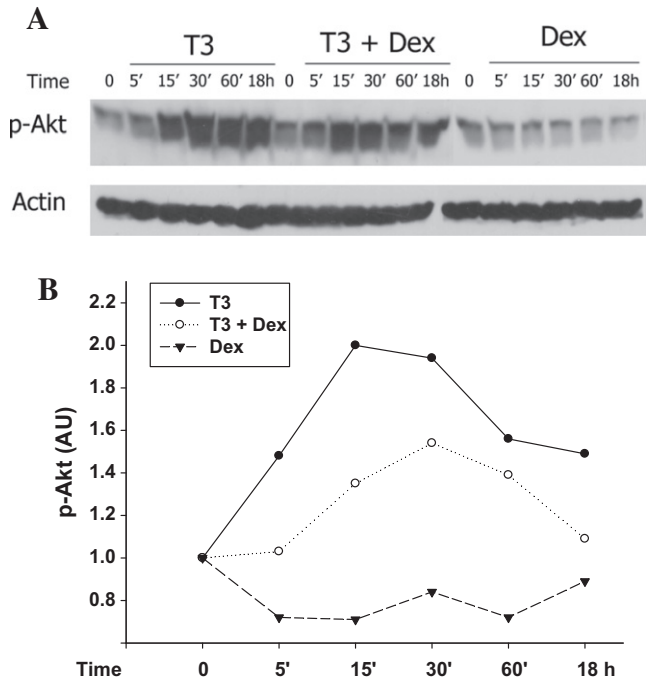

Fig. 5. Effect of Dex on T3-induced Akt phosphorylation on DCs. Bone marrow-derived iDCs differentiated with GM-CSF for 8 days were subjected to maturation with T3 (5 nM) in the absence or the presence of Dex (10 nM). After different time periods, cells were harvested and lysates were prepared and analyzed by Western blot for phosphorylated Akt (p-Akt). (**A**) Representative Western blot analysis of p-Akt is shown. Lower panel shows the same blot probed for actin to check equal loading of samples. (**B**) Results are expressed as arbitrary units (AU) obtained from the densitometric analysis of each p-Akt signal normalized to the actin signal in the same lane in one representative experiment from a total of three independent assays with similar results.

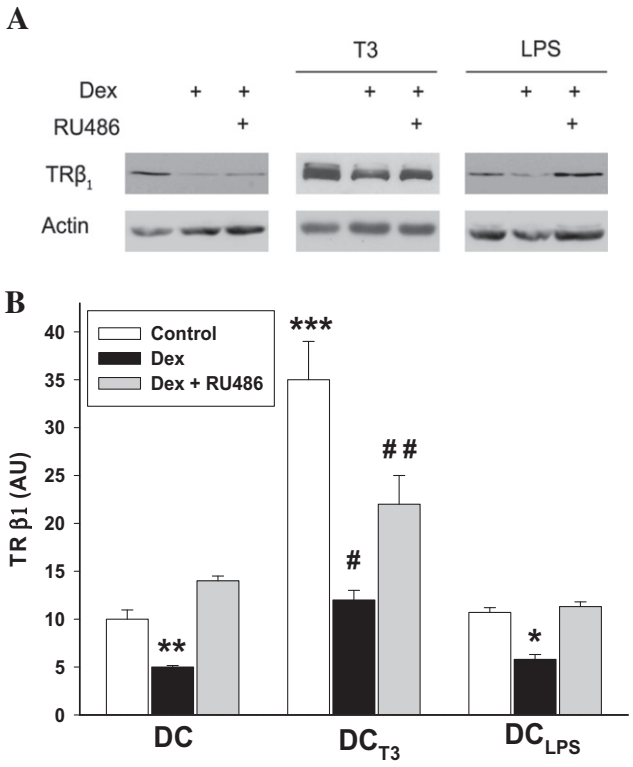

Fig. 6. Effect of Dex on $TR\beta_1$ expression on DCs. Bone marrow-derived iDCs differentiated with GM-CSF for 8 days (DC) were maturated by T3 (5 nM, DC_{T3}) or LPS (positive control, 100 ng/ml, DC_{LPS}) in the absence or the presence of Dex (10 nM) or Dex + RU486 (10 nM) (steroid hormone antagonist) for 24 h. (**A**) Representative Western blot analysis of protein extracts (40 μg) for immunodetection of $TR\beta_1$. Actin was used as a control of equal protein loading (lower panel). (**B**) Densitometric analysis of immunoreactive protein bands. Results are expressed as arbitrary units (AU) calculated from the densitometric analysis of each $TR\beta_1$ signal normalized to the actin signal in the same lane. Data are expressed as mean ± SD and are from one representative experiment from a total of three with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs DC Control, $^{\#}p < 0.001$ vs DC_{T3} Control, $^{\#\#}p < 0.05$ vs DC_{T3} + Dex.

effects, indicating that the inhibitory activity of Dex on T3 effects involves, at least in part, modulation of the NF-κB pathway.

4. Discussion

DCs are critical "decision-making" cells that must integrate signals from several pathways and receptors, including those arising from engagement of uptake and pattern recognition receptors, pro-inflammatory and anti-inflammatory cytokines, chemokines and hormones to determine the type and magnitude of adaptive immune responses [31]. Biological signals that control these cells can ultimately drive the direction of antigen-specific immune responses. GCs and THs are primary regulators of metabolic and endocrine processes, but they also have essential roles in immune and inflammatory responses [32,22]. Our study reports a major inhibitory role of GCs on the immunostimulatory capacity of T3-induced mature DCs and the molecular mechanisms underlying this effect.

Although the effects of Dex on LPS-activated DCs are well established [33,34] and the action of T3 and LPS in DC are exerted in a similar fashion, the mechanisms underlying T3-immunostimulatory effects do not involve the main signaling pathways activated by LPS [23,12,13]. Therefore, the effects of Dex on the biological activities of T3 cannot be predicted in advance. Exposure of iDCs to Dex considerably prevents the ability of T3 to promote DC maturation and drive T-cell activation and Th1 polarization. In fact, the MHC II and co-stimulatory molecules are substantially downregulated when mDCs are exposed to Dex. This effect is also reflected by the reduced frequency of $CD11c^{+}$ DCs producing IL-12, a potent pro-inflammatory cytokine responsible of generating Th1 cells which battle against intravesicular pathogens and tumors but also initiate autoimmune disorders. Interestingly, the ability of RU486 to counteract the inhibitory effects of GCs, strongly suggests the involvement of GR, disregarding unspecific membrane effects [35,2,36]. In turn and accordingly to the results presented, it is well known that GCs are potent inhibitors of LPS- or CD40L-induced phenotypic DC maturation and production of the pro-inflammatory cytokines IL-12p70 and TNF-α [37]. Moreover, RU486 prevented Dex-induced inhibition of TNF-α and IL-1β production by both rat bone marrow-derived and splenic DCs, indicating that GC effects on LPS-matured DCs are mediated through GR [34]. In agreement, corticosterone, the endogenous murine GC, functionally impaired DC maturation and cytokine production and reduced the ability of DC to prime naive $CD8^{+}$ T cells *in vivo*. This inhibition occurred via the GR with concentrations of corticosterone similar to those observed in animals undergoing stress responses [38].

We have previously found that, unlike LPS, T3 was not able to induce the synthesis of IL-10 by DCs [22]. Although Dex alone do not alter the synthesis of IL-10 in cultured DCs, this anti-inflammatory cytokine is increased in T3-conditioned DC cultures similarly to LPS-stimulated DCs following Dex treatment in agreement with previous reports [34,39]. These findings support the regulatory role of GCs in attenuating T3-dependent pro-inflammatory effects. Accordingly, we find an altered cytokine balance in the presence of Dex with an increased IL-10/IFN-γ ratio following co-culture of

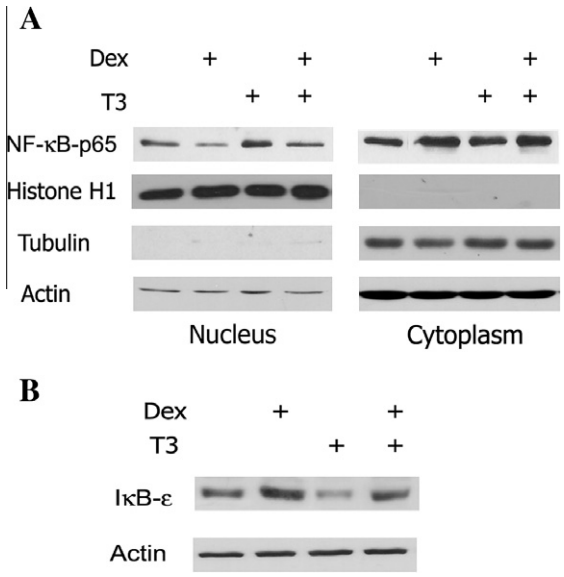

Fig. 7. Effect of Dex on cytoplasmic-nuclear shuttling of NF-κB-p65 and the levels of IκB-ε in T3-matured DCs. Bone marrow-derived iDCs differentiated with GM-CSF for 8 days were maturated by T3 (5 nM) in the absence or the presence of Dex (10 nM) for 18 h. (**A**) Representative Western blot analysis of nuclear and cytoplasmic DC extracts (40 μg) for immunodetection of NF-κB-p65. Anti-Histone H1 and tubulin Abs were used to control the purity of subcellular fractions. Actin was used as a control of equal protein loading (lower panel). (**B**) Representative Western blot analysis of protein extracts (40 μg) for immunodetection of IκB-ε. Actin was used as a control of equal protein loading (lower panel).

allogeneic T cells with T3-conditioned DCs (DC_{T3}). Taken together, these data demonstrate that DCs treated with Dex maintain an immature or tolerogenic state even in the presence of T3 where they preferentially secrete the immunomodulatory cytokine IL-10 rather than the pro-inflammatory counterpart IL-12, favoring T cell tolerance and suppression of host immunity [22]. In this regard, treatment of developing DCs with IL-10 promotes the generation of immature tolerogenic DCs that induce anergic $CD4^+$ and $CD8^+$ T cells *in vitro*, and regulatory $CD4^+$ T cells *in vivo* [40,41]. Interestingly, other groups have reported an altered DC-cytokine profile in the presence of Dex and other corticosteroids with increased IL-10 production [42,43,33] and a durable immature state, which could have been the result of high endogenous production of IL-10 [44]. Furthermore, Woltman et al. have shown that hyporesponsiveness and anergic states could be reversed by blocking both IL-10 and the inhibitory molecule programmed cell death ligand-1 (PD-L1) on DCs [45]. However, other studies showed that anti-IL-10 antibodies were unable to reduce DC induction of regulatory T cells [46], suggesting alternative mechanisms involved in this effect. In this regards, we have recently identified a tolerogenic circuit propagated from DCs to T cells by which galectin-1, a β-galactoside-binding lectin, drives the differentiation of tolerogenic DCs through mechanisms involving DC-derived IL-27 and T-cell derived IL-10 [47].

Consistently with our previous findings [23], exposure of DCs to T3 results in a rapid and sustained increase in Akt phosphorylation, which was essential for supporting T3-induced DC maturation and IL-12 production. In the present study, T3-induced Akt phosphorylation is rapidly inhibited in the presence of Dex and this inhibition lasts for several hours. These results may be the result of a rapid non-genomic activation and a parallel prolonged genomic regulation of GR-dependent mechanisms [48]. Accordingly, other authors reported the involvement of Akt activation in GC effects on human $CD4^+$ T cells [49] and chondrocytes [50].

Although we have demonstrated a physical interaction of GR with a consensus site for GC response element (GRE) present in the TRβ1 promoter that is responsible for Dex-induced TRβ1 expression in the liver [21], in the present study we find that Dex decreases TRβ1 expression in iDCs, LPS- and T3-maturated DCs. Since the magnitude of the cellular response to THs depends on the abundance and/or affinity of TRs, it is possible that alterations in the number or affinity of TRs as a result of physiologic or pathologic stimuli could modify tissue responses to T3 [51]. In this regard, Dex-induced inhibition of TRβ1 expression in DCs should be involved, at least in part, in GC-mediated impairment of the immunostimulatory capacity of T3-induced DCs. The opposite regulation of TRβ1 by Dex in the liver and DCs reinforces the tissue-specific actions of GCs [52]. Accordingly, previous studies reported an increase in vitamin D receptor (a hormone receptor with similar features and mechanisms of action as TR) by GCs in kidney, adypocytes, squamous cell carcinoma and human breast cancer cell lines whereas a considerable decrease was observed in intestinal mucosa [53,54]. In turn, the sensitivity and specificity of the GC response are determined by the complementary actions of individual GR isoforms that contribute to tissue- and cell-specific effects of GCs. The GR gene yields two distinct mRNAs by alternative splicing that result in the production of two GR isoforms termed GRα and GRβ. GRβ does not bind GCs, resides constitutively in the nucleus of cells, and acts as a dominant-negative inhibitor of GRα on genes both positively and negatively regulated by GCs. These GR isoforms show a widespread tissue distribution, but their relative levels differ both within and among tissues [52]. Although a similar GRα mRNA and protein expression in both human monocytes and iDCs has been reported, GRβ mRNA levels remained constant and very low throughout DC differentiation and maturation at 3–4 logs lower than that of GRα [55]. However, GR functionality is modulated at multiple levels that exceed GR isoform profile [56].

Previously, we have demonstrated an increase in cytoplasmic-nuclear shuttling of NF-κB-p65 after exposure of DCs to T3; this effect was associated with increased activity of the NF-κB pathway during T3-induced DC maturation and IL-12 production [22]. Furthermore, the immunostimulatory effects induced by T3 were significantly prevented by specific NF-κB inhibitors and NF-κB-siRNA [23]. The transcription of pro-inflammatory cytokines including IL-12p70 is controlled, at least in part, by the transcription factor NF-κB [57]. In this regard, GC-dependent repression of NF-κB-dependent transcriptional targets through direct physical association between GR and NF-κB in the nucleus has been consistently reported in several studies [58–60]. Moreover, inhibition of NF-κB activity by GCs has been proposed to be mediated by increased synthesis of IκBs, which sequester NF-κB in an inactive cytoplasmic form [61]. Hence, inhibition of T3-effects by Dex on DCs should be achieved, at least in part, through inhibition of NF-κB signaling. Furthermore, reduction of TRβ1 expression may also be explained by the presence of the functional consensus site for NF-κB located −644 to −652 bp up to the starting ATG transcription site of its gene recently reported by our group [23]. Therefore GC inhibition of the NF-κB pathway both at cytoplasmic and nuclear levels may lead to reduced NF-κB-dependent regulation of TRβ1 expression. Interestingly, other nuclear receptors also appear to be regulated by NF-κB; this includes the androgen receptor to which NF-κB specifically binds the −574/−565 promoter region and mediates repression of its transcriptional activity [62].

In conclusion, the results presented here reinforce the dynamic interrelationship between GCs and THs and their tissue-specific regulation and mechanisms of action, highlighting a novel molecular mechanism for the broad anti-inflammatory and immunosuppressive activities of GCs. In particular, our data reveals an inhibitory effect of GCs in T3-induced DC maturation and immunostimulatory capacity through mechanisms involving, at least in part, NF-κB and TRβ1 dependent signaling pathways. As these results were obtained in the presence of physiological T3

concentrations, these findings are relevant given the main role of THs in the initiation of immune responses, the widespread use of GC-based therapies and the prevalence of hypothyroid pathologies.

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